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Viroids: Survivors from the RNA World?

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Abstract

Because RNA can be a carrier of genetic information and a biocatalyst, there is a consensus that it emerged before DNA and proteins, which eventually assumed these roles and relegated RNA to intermediate functions. If such a scenario—the so-called RNA world—existed, we might hope to find its relics in our present world. The properties of viroids that make them candidates for being survivors of the RNA world include those expected for primitive RNA replicons: (*a*) small size imposed by error-prone replication, (*b*) high G + C content to increase replication fidelity, (*c*) circular structure for assuring complete replication without genomic tags, (*d*) structural periodicity for modular assembly into enlarged genomes, (*e*) lack of protein-coding ability consistent with a ribosome-free habitat, and (*f*) replication mediated in some by ribozymes, the fingerprint of the RNA world. With the advent of DNA and proteins, those protoviroids lost some abilities and became the plant parasites we now know.

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INTRODUCTION: WHY THE NEED FOR AN RNA WORLD?

The role of RNA has long been considered a subsidiary one, a mere link between DNA and proteins, which store and express genetic information in all cells. However, this is not true in the subcellular world, because the genetic material of many viruses—including that of *Tobacco mosaic virus* (TMV), the first discovered (4)—is RNA (48, 55). Although replication of some RNA viruses infecting bacteria (bacteriophages) was initially deemed to proceed through a mechanism different from that proposed for DNA (68), ensuing work with bacteriophage Qβ demonstrated that its single-stranded RNA (ssRNA) of about 4,200 nucleotides (nt) replicates by synthesizing a complementary strand via base pairing between A and U (instead of A and T, as in DNA), and G and C (41). Therefore, in these very simple (and presumably very old) self-replicating entities, RNA supplants DNA as the carrier of genetic information. Subsequent discovery of ribozymes, i.e., that some RNAs have catalytic activity (61, 81), showed that RNA can also express this information, supporting an early proposal that RNA could be the primordial macromolecule (18, 101, 143). This hypothesis was based on the ability of ssRNA to fold into complex structures—hairpins with stems stabilized by hydrogen bonds between bases and loops where the bases have no partner available—resembling those of proteins (50), and on its 2′ hydroxyl groups providing additional chemical versatility.

The term RNA world was coined in a commentary (56) following discovery of ribozymes:

> If there are ribozymes that can catalyze the synthesis of a new RNA molecule from precursors and an RNA template, then there is no need for protein at the beginning of evolution. One can contemplate an RNA world, containing only RNA molecules that serve to catalyze the synthesis of themselves. . . . At the next stage RNA molecules began to synthesize proteins, first by developing RNA adapter molecules that can bind activated amino acids, and then by arranging them according to an RNA template using other RNA molecules such as the RNA core of the ribosome. This process would make the first proteins, which

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> would simply be better enzymes than their RNA counterparts. . . . Finally, DNA appeared on the scene, the ultimate holder of information copied from the genetic RNA molecules by reverse transcription. After double-stranded DNA (dsDNA) evolved there exists a stable linear information store, error correcting because of its double-stranded structure but still capable of mutation and recombination. RNA is then relegated to the intermediary role that it has today—no longer the center of the stage, displaced by DNA and the more efficient protein enzymes.

This enlightening proposal has been extended into an entire field of research (3). Implicit in the RNA world idea is the existence of primitive RNA replicons of minimal size that later evolved into more complex self-replicating systems. Are there remnants of those minimal replicons in our present-day world? In the following sections we provide evidence that viroids (and some viroid-related replicons) are excellent candidates for being survivors from the RNA World. But first we summarize the unique, pertinent properties of these genetic elements. In-depth reviews dealing with different aspects of viroids have been published previously (30–32, 42, 43, 45, 66, 95, 128, 132).

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VIROIDS: ESSENTIAL FEATURES

Discovery of a Subviral World

A recurrent situation in science is that unanticipated discoveries entail unanticipated consequences going beyond the initial scheme. Viroids were discovered during an attempt to identify the agent of potato spindle tuber (PST) disease, suspected to be a virus. The finding that the PST agent can be transmitted to tomato, which is easier to grow and expresses symptoms more rapidly, expedited the experiments and produced the first surprise: After ultracentrifugation of tomato extracts, the infectious principle remained in the supernatant instead of being associated with the presumed viral particles—the virions, characteristically formed by protein subunits encapsidating the nucleic acid—expected to accumulate in the sediment. Besides, this infectious principle moved in sucrose gradient centrifugation more slowly than typical virions, and its mobility remained unchanged after deproteinating treatments, indicating that the PST agent might be a naked nucleic acid. This hypothesis was tested with polyacrylamide gel electrophoresis (PAGE): The PST infectious principle migrated as a band, absent in mock-inoculated plants, with the mobility predicted for a minuscule RNA (because of its sensitivity to RNase, but not to DNase). At this stage the term viroid was proposed for what appeared to be the first autonomously replicating subviral agent (*Potato spindle tuber viroid*, PSTVd) (26, 27). Application of this methodology quickly resulted in discovery of novel viroids as the causal agents of other plant diseases of presumed, but not proved, viral etiology. Altogether these studies opened a window onto a subviral world composed of small, self-replicating RNAs, displacing viruses from the lowest step of the biological scale, which they had occupied during the first 65–70 years of the twentieth century. It is worth noting that the subviral world, like the viral world, was discovered by studies on plant systems. However, in contrast to viruses, viroids so far have been restricted to the plant kingdom, although at least one viroid-related replicon is involved in a human disease (see below).

Structure: Small Circular RNAs with Compact Folding

First of all, how small was PSTVd compared with the RNAs of known viruses? Early estimations from PAGE, corroborated by electron microscopy of purified PSTVd RNA (124), were about 300 nt, a size at least 10-fold smaller than that of the ssRNAs of TMV (6,400 nt) and bacteriophage Qβ (see above). Moreover, electron microscopy of denatured PSTVd RNA produced a

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second surprise: The RNA was circular, a feature previously conjectured given its resistance to exonucleases. Together, the small size (the RNA encoding, if any, translation products of minimal complexity) and circularity (which hampered translation by conventional ribosome scanning from a 5′ terminus) suggested that PSTVd was a nonprotein-coding RNA. Determination of its 359-nt primary structure by direct RNA sequencing (a milestone in molecular biology, given that it was the first eukaryotic pathogen completely sequenced) confirmed the circular structure and the absence of typical initiation codons (59). From a functional perspective this was a distinctive point, because all viruses encode proteins mediating one or more steps of their biological cycle. Furthermore, thermodynamic predictions (109), along with RNase and bisulphite probing in vitro, supported the idea of intramolecular folding of PSTVd into a rodlike secondary structure—also favored by circularity and composed of double-stranded segments flanked by apparently unstructured loops—with a width akin to that of a dsDNA as observed by electron microscopy under nondenaturing conditions.

Sequencing of other viroids confirmed preservation of the rodlike folding and, within it, of a central conserved region (CCR) (90), which emerged as an idiosyncratic trait (**Figure 1**). However, this was not the case of *Avocado sunblotch viroid* (ASBVd) (126), which did not have a CCR but had strands of both polarities displaying a remarkable behavior: They self-cleaved through

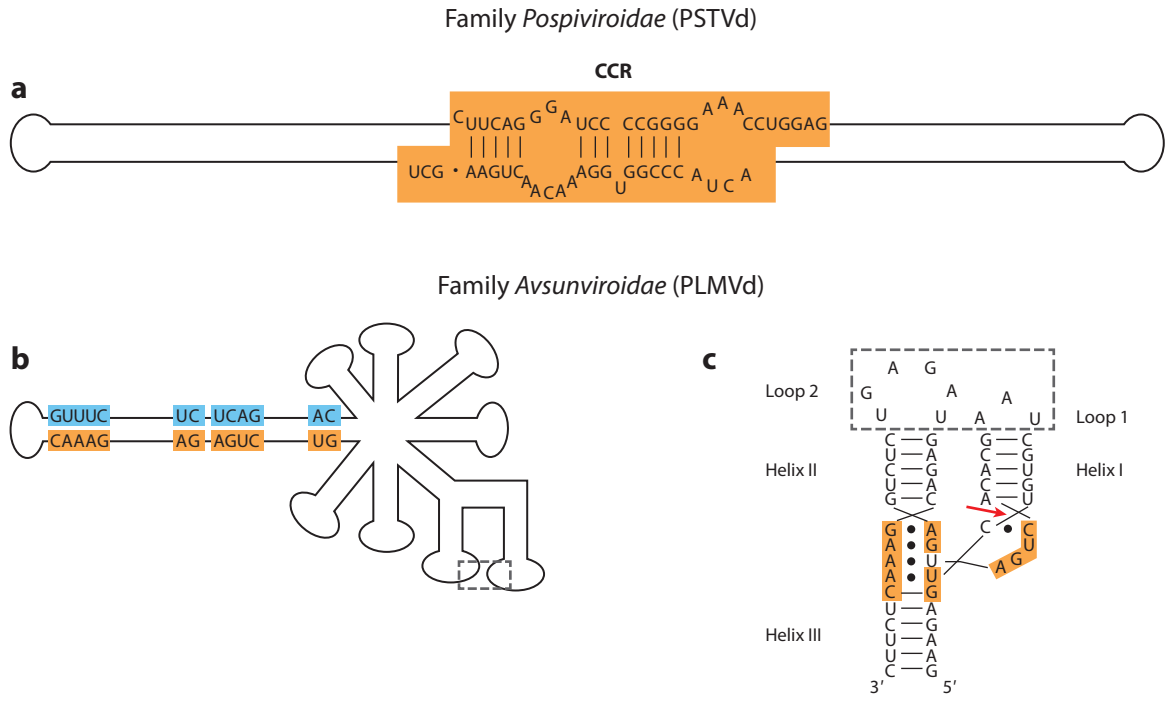


Figure 1

Viroid structure. (*a*) Rodlike secondary structure with a central conserved region (CCR) proposed for PSTVd (family *Pospiviroidae*). (*b*) Branched secondary structures proposed for PLMVd (family *Avsunviroidae*) and for its plus-polarity hammerhead ribozyme. (*b*,*c*) Nucleotides strictly or highly conserved in natural hammerhead structures are shown within boxes with orange and blue backgrounds for plus and minus polarities, respectively. Continuous lines and dots between nucleotides represent canonical (Watson-Crick) and noncanonical base pairs, respectively, with the red arrow marking the self-cleavage site. Dashed rectangles denote tertiary interactions between loops that either stabilize the global viroid conformation or promote the catalytically active ribozyme folding.

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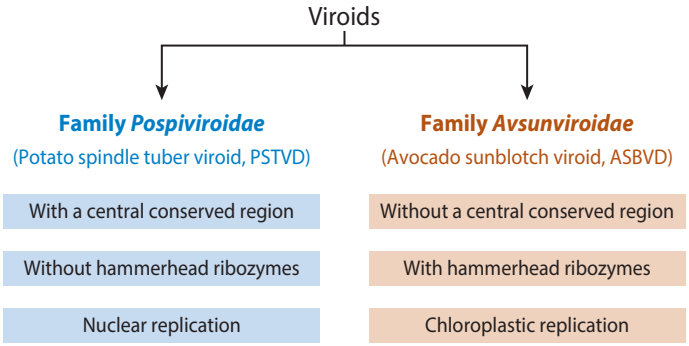


Figure 2

Viroid classification. Distinctive structural and functional features of the two viroid families, *Pospiviroidae* (from POtato SPIndle tuber VIROID) and *Avsunviroidae* (from AVocado SUNblotch VIROID).

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hammerhead ribozymes, thus acting as catalytic RNAs (74), an attribute with deep implications for its replication and evolutionary origin. PSTVd and ASBVd epitomized two viroid groups, which ultimately resulted in recognition of the families *Pospiviroidae* and *Avsunviroidae*, respectively (37, 43), a cataloging consistent with other distinctive features that include the subcellular site and mode of replication (see below) (**Figure 2**). Moreover, some members of the *Avsunviroidae*, like *Peach latent mosaic viroid* (PLMVd) (71) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) (93), fold into branched secondary structures (**Figure 1**).

Replication: Rolling-Circle Mechanism Catalyzed by Enzymes and Ribozymes

Because viroids are nonprotein-coding RNAs, they need to usurp the transcription and processing machinery of their hosts to replicate. Thus, viroids are essentially transcriptional parasites, whereas viruses are essentially translational parasites (to infect hosts, they must express the proteins they encode). Viroid replication takes place in the nucleus (family *Pospiviroidae*) or in plastids, mostly chloroplasts (family *Avsunviroidae*), through a rolling-circle mechanism with RNA intermediates (7, 21, 58, 73). The infecting circular (+) RNA (the sign is conventionally allocated to the most abundant strand in vivo) is repeatedly transcribed into oligomeric (−) strands. These replicative intermediates, by themselves (asymmetric pathway) or after being cleaved and ligated into circular monomers (symmetric pathway), serve as templates for synthesis of oligomeric (+) strands that are finally processed into their monomeric circular counterparts (**Figure 3**).

Therefore, three enzymes (or ribozymes) are required: RNA polymerase, RNase, and RNA ligase, the characterization of which has led to unexpected discoveries. First, despite the existence of several RNA-dependent RNA polymerases in plants (118, 138), transcription of viroid strands is catalyzed by nuclear or plastidic DNA-dependent RNA polymerases forced to accept RNA templates (44, 92, 97, 113, 119). Second, cleavage of oligomeric strands is mediated in the family *Pospiviroidae* by a class III RNase enzyme that operates on a dsRNA structure formed by the upper CCR strand of two consecutive units (53), or by *cis*-acting hammerhead ribozymes in the family *Avsunviroidae* (74). And third, circularization occurs by a plastidic isoform of the tRNA ligase (99) or, remarkably, by nuclear DNA ligase 1 (redirected to circularize RNA substrates) (98). Therefore, manifesting their extraordinary parasitic abilities, viroids reprogram the template specificity of a DNA-dependent RNA polymerase to function as an RNA-dependent RNA polymerase, and the substrate specificity of a DNA ligase to operate as an RNA ligase. To complete their infectious

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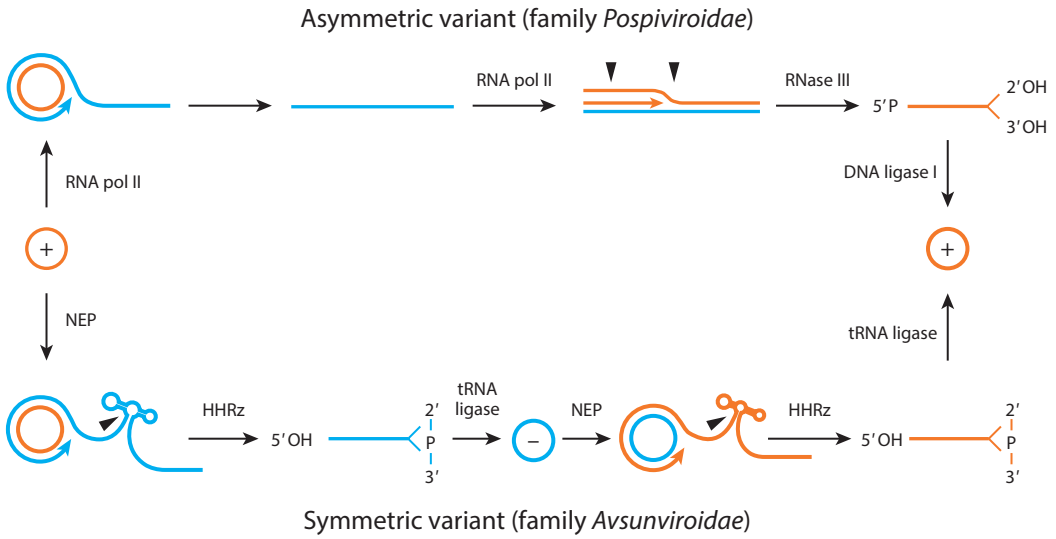


Figure 3

Asymmetric and symmetric variants of the rolling-circle mechanism proposed for replication of members of the families *Pospiviroidae* and *Avsunviroidae*, respectively. Orange and blue colors refer to plus and minus polarities, respectively, with cleavage sites denoted by arrowheads. The enzymes and ribozymes that presumably catalyze the replication steps are indicated. Notice that RNA polymerase II (and NEP) is redirected to transcribe RNA templates and DNA ligase 1 to circularize RNA substrates. Abbreviations: HHRz, hammerhead ribozyme; NEP, nuclear encoded polymerase.

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cycle, viroids also need to move to invade distal plant parts. Recent data indicate that specific loops/bulges in the rodlike structure of PSTVd and related viroids are, instead of unstructured, stabilized by arrays of noncanonical pairs, with some of these elements functioning in replication and others in systemic trafficking (31, 32, 147).

Sequence Diversity

As a consequence of their high mutation rates (see below), some viroids display high genetic variability. This phenomenon has been observed after inoculating plants with infectious viroid cDNAs (usually head-to-tail dimeric constructs) or their transcripts (mimicking replicative intermediates) and quantifying the heterogeneity of the resulting progeny. The diversity observed in the family *Pospiviroidae*, e.g., in PSTVd (57), *Citrus exocortis viroid* (5, 64, 135), *Citrus bent leaf viroid* (52), *Chrysanthemum stunt viroid* (CSVd) (16), and *Citrus dwarfing viroid* (131), is relatively low, and the consensus sequence does not deviate significantly from the wild type. However, in the family *Avsunviroidae*, e.g., PLMVd (2, 94) and CChMVd (16, 23), the resulting population is a complex spectrum of mutants and the consensus sequence often changes over time or is difficult to define. This difference is most likely caused by the distinct replication fidelity of the RNA polymerases involved (see below), with selection imposed by the host also shaping the final distribution of genetic variants (5, 122, 131, 139).

Given their small genomes and high mutation rates, viroids have often been treated in the framework of the quasi-species theory, originally developed to describe natural selection acting on primitive RNA replicons undergoing error-prone replication and consisting of mutant distributions (mutant spectra or clouds) (6, 34). This theoretical scheme was first applied to

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bacteriophage Qβ (33) and then to many other RNA viruses (84, 100, 134). A unique prediction of the quasi-species theory that distinguishes it from classic population genetics models is that selection operates on sets of variants from a particular region of the fitness landscape rather than on individual virus variants. As a result, the fitness associated with a specific sequence depends on the average fitness of its neighbors in the sequence space (84), as first experimentally shown with the RNA bacteriophage ϕ6 (11). This collective behavior can lead to the so-called survival of the flattest effect, whereby a population located in a region of the fitness landscape where a large proportion of sequence neighbors are selectively neutral can outcompete another population located in a higher-fitness peak but with a more deleterious sequence neighborhood (121, 141). This effect was first demonstrated in vivo by competition experiments between CSVd and CChMVd, performed under different conditions: At the physiological mutation rate the fittest CSVd outcompeted CChMVd, but increasing mutation rate by UV irradiation reversed the outcome and the flattest CChMVd outcompeted CSVd (16). Later, the survival of the flattest was also shown in *Vesicular stomatitis virus* populations subjected to increased mutational stress by chemical mutagenesis (115).

Recent work has suggested that interactions such as genetic complementation and interference among individual components of the viral population also determine to a good extent its behavior, with interference most likely being exerted by defective gene products resulting in nonfunctional protein complexes during the virus biological cycle (100). However, since viroids are nonprotein-coding RNAs, complementation by protein products acting in *trans* cannot exist, and interference must occur at the RNA level.

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VIROID-RELATED REPLICONS

Viroid-Like Satellite RNAs

Besides viroids, three other viroid-related subviral elements deserve mention in the context of the RNA world. Plant viroid-like satellite RNAs are structurally similar to viroids, comprising only a small nonprotein-coding circular RNA (114, 120, 127). Additional similarities include replication through a rolling-circle mechanism with ribozyme-mediated self-cleavage of the resulting RNA oligomeric strands of one or both polarities. However, ribozymes are not only of the hammerhead class (46, 106), but also of the hairpin class (12). More important, replication of viroid-like satellite RNAs is not autonomous but relies on the RNA replicase complex encoded—at least in part—by a coinfecting helper RNA virus (whose coat protein encapsidates the satellite RNA for transmission); the term satellite refers to this dual dependence (10). Because the helper RNA viruses replicate in organelle-associated membranous vesicles connected with the cytoplasm (25), replication of viroid-like satellite RNAs most likely also takes place in these vesicles, although lack of direct supporting data leaves open the possibility of the helper virus encoding protein(s) that redirect(s) a host RNA polymerase (127). Resembling the situation found in the family *Avsunviroidae*, circularization of unit-length strands resulting from self-cleavage in viroid-like satellite RNAs might be mediated by a cytoplasmic isoform of the tRNA ligase, or by self-ligation (the RNA ligase activity of the hairpin ribozyme is greater than that of the hammerhead ribozyme) (12, 40).

Retroviroid-Like Elements

A second class of plant small circular RNA is similar to viroid and viroid-like RNAs in size and in harboring hammerhead ribozymes (70) but has a singular property: The RNA is accompanied by a homologous DNA counterpart fused to sequences of either a pararetrovirus or the host (19, 69). This class, dubbed retroviroid-like elements because the homologous DNA is presumably

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generated by a reverse transcriptase (RT)—pararetroviruses encode such an enzyme—is restricted so far to some carnation sources, and it cannot be transmitted horizontally. The presence of hammerhead ribozymes in both polarity strands of this carnation circular RNA is evidence of its replication through a symmetric rolling-circle mechanism, but the lack of horizontal transmission suggests that a supply of transcripts from the DNA form is needed.

Hepatitis δ Virus

In contrast with the other two, the third class of viroid-related subviral entities has been reported in animals, specifically in humans. With an approximate size of 1,680 nt, the *Hepatitis δ virus* (HDV) RNA—the smallest genome of an animal virus—occupies the position immediately above viroids on the biological scale of genome sizes, and it displays striking structural similarities with viroids: circularity and folding into a rodlike secondary structure (15, 110, 111, 136). Moreover, HDV RNA replicates autonomously in the nucleus through a rolling-circle mechanism catalyzed by host enzymes (an RNA polymerase redirected to transcribe RNA templates and, presumably, an RNA ligase) and by *cis*-acting ribozymes idiosyncratic to this infectious agent (82, 83). However, HDV RNA encodes in its antigenomic polarity a protein (like some RNA viruses) (136), and for transmission it depends on a helper virus, *Hepatitis B virus* (similar to viroid-like satellite RNAs).

WHY ARE VIROIDS AND VIROID-RELATED REPLICONS REGARDED AS SURVIVORS OF THE RNA WORLD?

Early Speculations on the Origin of Viroids

Initial ideas suggested that viroids could be escaped introns, thus descending from host RNAs. This proposal was based on (*a*) sequence similarities of PSTVd (and other viroids) with group I and II introns, and (*b*) specific cleavage of viroid oligomeric replication intermediates and their following ligation into circular forms, conceptually resembling cleavage-ligation by which introns are self-spliced (and circularized) from primary transcripts and exons are joined into mature RNAs (28, 63). Yet, subsequent findings that reaction mechanisms were distinct eroded the credibility of the viroid-intron connection.

Moreover, comparative sequence analysis of PSTVd and two related viroids revealed conspicuous similarities with the ends of transposable elements, which together with the presence of inverted repeats and flanking imperfect direct repeats suggested that viroids might have originated from transposable elements or retroviral proviruses by deletion of internal fragments (79). However, despite initial reports indicating the presence of PSTVd sequences in cellular DNA of normal plants, further analyses failed to confirm these results, and moreover, ASBVd (and later other members of its family) did not fit this model. Therefore, viroids do not seem to be simplified versions of viruses or transposons.

Circular RNAs: Relics of Precellular Evolution?

With this provoking title, Diener (29) gave a twist to previous perspectives and proposed that viroids and viroid-like satellite RNAs were more likely candidates than introns as living fossils of a precellular RNA world—precellular should be understood to mean lacking cells as they are known today—and he showed that their characteristic features might have evolved to cope with barriers in the self-replication of primitive RNAs. Further studies have reinforced this view and

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VIROIDS: THE OLDEST RNA REPLICONS?

Characteristics of viroids giving evidence that they may have a very old origin:

- Minimal genomes (250–400 nucleotides)
- High G + C content, increasing replication fidelity of primitive RNA polymerases
- Circular structure, excluding need of genomic tags for complete replication
- Structural periodicities facilitating modular assembly
- No protein-coding capacity, suggesting emergence before the ribosome
- Presence of ribozymes, the signature of the RNA world

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filled some gaps. Hereafter we discuss the evidence that supports Diener's claim, summarizing his arguments and supplying others (See sidebar, Viroids: The Oldest RNA Replicons?).

First, the intrinsic error-prone replication of primitive RNA systems imposed a limit to the size of their master (predominant) sequences to overcome the "error catastrophe" leading to extinction (34). Thus, the evolutionary paradigm that the simplest is the oldest, which with some limitations is generally accepted, seems appropriate for viroids because they are the smallest known replicons.

Second, to increase replication fidelity, (G + C)-rich sequences would have been selected in primitive RNA systems because of the greater thermodynamic stability of GC pairs versus their AU counterparts (35). Viroids also meet this requirement: they are (G + C)-rich with a notable exception, ASBVd (126). Recently, it was proposed that the conflict intrinsic to the RNA world between stable folding (required for ribozyme activity) and template ability (required for replication) could have been solved by a division of labor between the two RNA strands, with one (containing wobble G:U pairs) folding into a ribozyme and the reverse complement (containing the less stable C:A mispairs) functioning as genome (75). Analysis of 40 viroid sequences is consistent with this proposal, a remarkable finding because they must have evolved in response to different selection pressures beyond a simplified division of labor (75). However, this proposal should be restricted to members of the family *Avsunviroidae*, the only ones with hammerhead ribozymes. Furthermore, given that the ribozymes can be formed by the two strands and both function as templates (**Figure 3**), the division of labor view becomes blurred.

Third, circularity allows complete replication without recurring genomic tags, which mark specific initiation sites in linear templates and ensure their end-to-end copying. Even if polymerization of viroid strands in the present cellular habitat starts at defined sites (24, 80, 96), this may not have occurred in primitive replicons, for which reiterative copying of circular genomes—irrespective of initiation at a defined position—would avoid loss of genetic information. Moreover, this rolling-circle replication would result in multiple copies of the genetic information, a condition likely favored in primitive replicons with a high mutation rate (107). Inherent to this replication mode is the need for a processing mechanism (cleavage and circularization) of the oligomeric replication intermediates (**Figure 3**). Members of the family *Avsunviroidae* fulfill the first step of this mechanism, because their oligomeric strands self-cleave through hammerhead ribozymes; the second step also appears feasible, as revealed by the high level of RNA ligase activity associated with the hairpin ribozymes present in some viroid-like satellite RNAs (10, 12).

Fourth, the sequence of some viroids exhibits a structural periodicity characterized by repeat units of different length. Although this periodicity was initially associated with the protein-binding

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ability of viroids (78), other interpretations pointed to a mechanism by which larger genomes could have evolved (29). Even if the strength of this argument is weakened by the lack of structural periodicity in at least one viroid and in most viroid-like satellite RNAs (29), the idea that viroids could have been built modularly seems attractive, particularly if several instead of a single module are considered. The recombinant nature of some viroids, which are chimeras formed by fragments present in other viroids, is consistent with this view (67, 108). Moreover, modular evolution is more advantageous than direct evolution of large functional molecules because of the higher mutation rates allowed, the shortening of evolutionary times, and the possible emergence of complex structures that could not be otherwise directly selected (88). According to computational predictions the module repertoire would be dominated by hairpin-like structures, a fraction of which could have RNA ligase activity and catalyze assembly of larger, eventually functional RNAs; this stepwise ligation-based model of modular evolution could ease the path to the appearance of a ribozyme with RNA replicase activity (9).

Fifth, viroids are nonprotein-coding RNAs. This key feature not only distinguishes viruses from viroids but also traces their putative origin back to what has been called the first age of the RNA world. This first age was delimited between the emergence of the first replicating RNA cell(s)—a metabolism without cellular confinement seems unlikely—and just before the advent of the ribosome, which opened the second age of the RNA world; this second age lasted until the appearance of the first replicating DNA cell(s), which eliminated their parental RNA cell counterparts in Darwinian evolution (47). Even the catalytic component of present-day ribosomes is RNA, with proteins just providing a scaffold: "The ribosome is a ribozyme," as has been vividly summarized (13). Thus, viroids comply with the lack of mRNA activity expected for a replicating relic of the (first age of the) RNA world, wherein no ribosomes existed.

Sixth and most important, some viroids display catalytic activity via hammerhead ribozymes. When Diener formulated his hypothesis (1989), ribozymes (the key signature of the RNA world) were known to be prevalent among viroid-like satellite RNAs but restricted to just one (and peculiar) viroid (ASBVd). Subsequent discovery of three additional viroids with hammerhead ribozymes in both polarity strands, PLMVd (71), CChMVd (93), and *Eggplant latent viroid* (39)—and more recently of two other candidates (144, 146)—dispelled the doubts that ASBVd could be a viroid-like satellite RNA and consolidated the family *Avsunviroidae*. The small size and relatively low structural complexity of the hammerhead and hairpin ribozymes, much simpler than those mediating intron splicing, also support their involvement in self-replication of RNA. The hammerhead and hairpin ribozymes can catalyze two of the three steps of viroid replication: cleavage and ligation, but not polymerization (**Figure 3**). RNA-catalyzed RNA polymerization has not been reported in nature, but in vitro evolution studies have shown that RNA can catalyze this reaction (77, 87, 142), thus making viroid replication in a preprotein world feasible.

Do viroids have a monophyletic origin? The low overall sequence similarity among these RNAs poses a problem for inferring a reliable phylogeny (76). Nevertheless, when alignments are adjusted—considering the local similarities and the insertions/deletions and duplications/rearrangements described—and an appropriate estimator of genetic distances is used, phylogenetic reconstructions support a monophyletic origin. Furthermore, these reconstructions are consistent with the major groups proposed previously for viroids (and viroid-like satellite RNAs), as well as with their biological properties (36, 37). Yet, a polyphyletic viroid origin cannot be dismissed, particularly considering the unusual base composition of ASBVd. Furthermore, thermodynamic predictions of the secondary structure of viroid RNAs support an evolutionary trend during viroid radiation toward increased robustness (resistance to deleterious mutations) and reduced antagonistic epistasis (interactions between deleterious mutations) (116, 117).

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Transition from the RNA World to a World with Proteins and DNA: Viroids

Because nearly all intermediate stages have declined with time, it is difficult to speculate how viroids evolved from the RNA world, more than 3,000 mya, into a world involving two additional macromolecules (proteins and DNA). Nevertheless, at least some hints can be inferred from a close examination of these small replicating RNAs. Focusing on the distinction between families *Pospiviroidae* (or nuclear viroids) and *Avsunviroidae* (or chloroplastic viroids), two reasons argue in favor of the latter preceding the former. According to the endosymbiont hypothesis, plastids derive from primitive cyanobacteria by symbiosis (89); these free-living cells, which emerged prior to eukaryotic cells, might have hosted (and perhaps still host) the ancestors of the family *Avsunviroidae* before they adapted to plants (14, 86). In support of this view, the mediation of one step of the replication cycle by the hammerhead ribozyme has been retained only in the family *Avsunviroidae*. Conceivably, the most complex nuclear environment provided increased opportunities for the complete transition from RNA- to protein-catalyzed replication, whereas this transition has been partially accomplished in plastids. Yet, it is intriguing that the two families have adopted the same strategy regarding polymerization of RNA strands: to redirect DNA-dependent RNA polymerases to transcribe RNA templates. Although such a scenario may appear realistic for viroids replicating in plastids, wherein no RNA-dependent RNA polymerase has been described, the situation is different for viroids replicating in the nucleus, wherein several enzymes of this class accumulate. An alternative and more plausible explanation is that the DNA-like compact folding of the genomic viroid RNA (and its replicative intermediates) may have facilitated their recognition by RNA polymerases that transcribe double-stranded templates into ssRNAs, in contrast to RNA-dependent RNA polymerases that recognize ssRNA templates and convert them into dsRNA products.

On the one hand, two DNA-dependent RNA polymerases reside in plastids (1, 125), and the available evidence supports the involvement of one of them, the nuclear-encoded polymerase (NEP), in replication of members of the family *Avsunviroidae* (97, 113). This finding is consistent with the NEP, comprising a single subunit, being structurally simpler (and presumably older) than the plastid-encoded polymerase (PEP), comprising five subunits. On the other hand, the three nuclear DNA-dependent RNA polymerases typical of eukaryotes (RNA polymerases I to III) (17), and the two additional ones described recently in plants (RNA polymerases IV and V) (62), display a high level of structural complexity (with more than 10 subunits). Why members of the family *Pospiviroidae* have specifically co-opted RNA polymerase II (44, 92, 119) remains an enigma, but interestingly, the same enzyme is also involved in HDV RNA replication (129). Perhaps this aptitude of RNA polymerase II is related to its versatility in catalyzing transcription of protein-coding genes as well as long and small noncoding RNAs (62).

The RNA polymerases mediating replication of viroids are also the driving force that largely determines two of their features: the differential complexity of viroid quasi-species and the recombinant nature of some viroids. The mutation rate estimated for CChMVd, 0.0025 per site and replication cycle (one mutation per replicated genome), is the highest reported for any biological entity (**Figure 4**) (51). This extremely error-prone replication—most likely caused by a proofreading-deficient RNA polymerase (NEP) forced to use RNA instead of its native DNA template (43) and perhaps also by accumulation of mutagenic free radicals in plastids or unbalanced nucleotide pools—is reminiscent of that postulated for the primitive replicons of the RNA world. Viroids, and more explicitly members of the family *Avsunviroidae*, can possibly withstand this elevated mutation rate because they have a very small genome, whereas more complex genomes would accumulate an excessive mutational load (34). These results also suggest that emergence of mechanisms enhancing replication fidelity was critical for the evolution of complexity in the early

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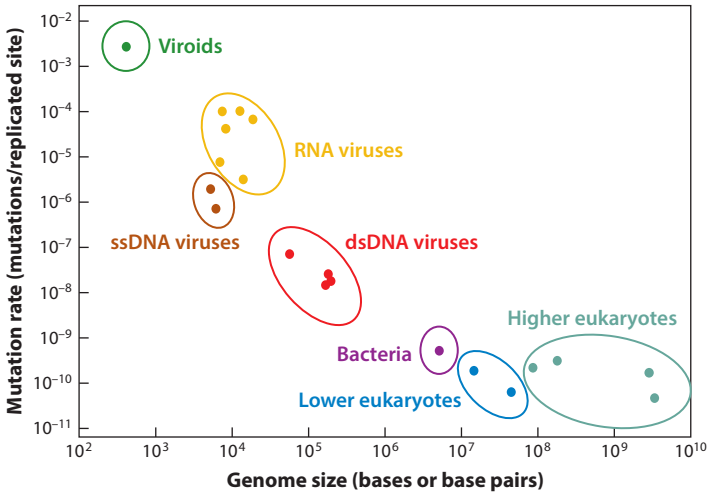


Figure 4

Per-site mutation rate versus genome size for *Chrysanthemum chlorotic mottle viroid* and other biological entities. RNA viruses (*left* to *right*) are *Tobacco mosaic virus*, human rhinovirus, poliovirus, *Vesicular stomatitis virus*, bacteriophage ϕ6, and *Measles virus*; all are single stranded except bacteriophage ϕ6, which is double stranded. Single-stranded DNA (ssDNA) viruses are bacteriophage ϕX174 and bacteriophage m13. Double-stranded DNA (dsDNA) viruses are bacteriophage l, *Herpes simplex virus*, bacteriophage T2, and bacteriophage T4. Bacteria are *Escherichia coli*. Lower eukaryotes are *Saccharomyces cerevisiae* and *Neurospora crassa*. Higher eukaryotes are *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens*. Where several estimations were available, the mean value is shown. Reproduced with permission from Reference 51.

history of life (51). Furthermore, the need to transcribe atypical viroid RNA templates might also decrease the processivity of the otherwise DNA-dependent RNA polymerases, promoting their stalling and subsequent jumping with the bound nascent transcript, thus facilitating the appearance of chimeric viroids. Their recurrent identification (133) supports the view that recombination has played an important role in shaping the viroidsphere.

The other two replication steps further attest to the resilience of viroids. For cleavage of their oligomeric replication intermediates, members of the family *Pospiviroidae* recruit RNases of class III mediating posttranscriptional maturation of highly structured nuclear transcripts (53). Members of the *Avsunviroidae* recruit RNA-binding proteins with a similar role in processing of chloroplastic transcripts, which additionally behave as RNA chaperones that in vitro, and presumably in vivo, stimulate the efficiency of hammerhead ribozymes (20). For circularization of the resulting unit-length strands, PSTVd (and most likely the other members of the family *Pospiviroidae*) divert DNA ligase 1 to function as an RNA ligase, taking advantage of the fact that, upon DNA binding, DNA ligase 1 promotes a local RNA-like A-form conformation in the vicinity of the DNA break (102, 103). Moreover, DNA ligase 1 and some RNA ligases, like T4 RNA ligase 1, demand 5′-phosphomonoester and 3′-hydroxyl termini like those generated by class III RNases and operate through a similar mechanism (72, 137). Further supporting this view, DNA ligases, RNA ligases, and RNA-capping enzymes probably evolved by fusion between auxiliary effector domains and an ancestral catalytic module mediating RNA repair (123). Thus, some viroids would opportunistically exploit the preservation of a primordial role of DNA ligase 1 (98). In contrast, for ligation of the (+) and (−) monomeric linear replication intermediates, members of the family *Avsunviroidae* recruit a chloroplastic tRNA ligase isoform with specificity for the 5′-hydroxyl and 2′,3′ cyclic phosphodiester termini produced by hammerhead ribozymes (99).

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Transition from the RNA World to a World with Proteins and DNA: Viroid-Related Replicons

The features of viroid-like satellite RNAs, both structural (small size and circularity) and functional (lack of mRNA ability and replication by a rolling-circle mechanism mediated by hammerhead and hairpin ribozymes), support a common origin with viroids and, particularly, with members of the family *Avsunviroidae* (36). However, in their subsequent evolution, viroid-like satellite RNAs have followed a different strategy: Instead of relying exclusively on the host transcriptional and processing machinery, they have additionally parasitized a helper virus that provides the transmission and most likely part of the replication machinery. This ménage à trois, more complex but possibly more efficient for ensuring dispersal of the viroid-like RNAs by the helper virus—viroids are self-sufficient in this respect—also suggests that parasitism emerged very early in the history of life.

Resembling viroid-like satellite RNAs, HDV has also evolved to parasitize a helper virus, but only for transmission because HDV RNA, like viroids, replicates autonomously in infected cells. How HDV RNA might have appeared has been discussed in extenso previously (130). Here we just emphasize that analysis of HDV RNA has uncovered its recombinant nature: The rodlike secondary structure can be regarded as the fusion of a viroid-like domain, holding the ribozymes and located in one of the terminal domains, to another domain that in the antigenomic polarity contains the open reading frame for the so-called δ antigen (δAg), a protein with a crucial role in the biology of the virus (60). This modular design has led to the proposal that HDV RNA might have arisen from a self-replicating, viroid-like RNA capturing the mRNA encoding a protein, like the δAg, which, having a beneficial effect on the recombinant product, would have secured its persistence (8, 112).

In contrast to viroids and the other viroid-related replicons, retroviroid-like elements have a DNA counterpart and, therefore, should have appeared following the "invention" of this biopolymer. DNA can be viewed as a derivative of RNA, since all organisms synthesize deoxyribonucleotides by reducing ribonucleotides, and thymine by methylating uracil (85). The first of these two reactions, due to its complex sulfur-based mechanism, seems unlikely to have been catalyzed by a ribozyme, thus demanding a protein enzyme. This argument and others (49, 85) support the view that DNA appeared during the second age of the RNA world (after the advent of the ribosome) (47). RT, mediating transition from RNA to DNA, most likely evolved from an ancestral RNA-dependent RNA polymerase—ribozyme or protein enzyme—involved in replication of early RNA genomes (91, 104, 145). By acquiring a homologous DNA from which RNA can be transcribed, retroviroid-like elements would have increased their chances for survival. Intriguingly, following initial discovery of hammerhead ribozymes in certain newt transcripts (38), recent studies have unveiled the existence of hammerhead and HDV-like ribozymes along the biological scale, with the insertion site of the corresponding DNAs in the host genomes indicating a possible regulatory function and the contribution of retrotransposition to their genesis (22, 65, 140). However, it is unclear whether the RNA products resulting from ribozyme-mediated self-cleavage are subsequently circularized.

SUMMARY

The time had clearly come to ask how the DNA→ RNA→protein flow of information had ever gotten started. Here, Francis [Crick] was again far ahead of his time. In 1968 he argued that RNA must have been the first genetic molecule, further suggesting that RNA, besides acting as a template, might also act as an enzyme and, in so doing, catalyze its own self-replication.

—J.D. Watson (54, p. xxiii)

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The course of time has provided accumulating evidence to this passionate laudation by J.D. Watson in the prologue of the first edition (54) of the classic book on the RNA world (3). Just a recent example: The synthesis of activated pyrimidine ribonucleotides starting from plausible prebiotic precursors and conditions has bypassed the difficulty of how ribonucleotides could have been formed from their constituent parts (ribose and nucleobases) and removed another important hurdle for the emergence of the RNA world (105). The survival until today of viroids (and viroid-related replicons) adds further support to this view.

1. Viroids fulfill all structural criteria postulated for primitive replicons, including small size, (G + C)-rich content and compact folding, as well as circularity that may ensure complete replication and even polyploidy.
2. From a functional perspective, viroids lack protein-coding ability, but at least some encode ribozymes; in other words, they are catalytic RNAs, the key signature expected for survivors of the RNA world.
3. The three catalytic activities mediating rolling-circle replication of viroids, namely, RNA polymerization, cleavage, and ligation, can be supplied by natural or artificial ribozymes, making replication reasonable in a protein-free world.
4. Viroid-like satellite RNAs, retroviroid-like elements, and HDV RNA can be regarded as evolutionary inventions related to (and possibly derived from) viroids.
5. Feasible evolutionary routes can be envisaged for the transition of viroids and viroid-related replicons from the RNA world to a world with proteins and DNA.

Science runs through winding roads. The initial purpose of characterizing the viruses presumed to cause several plant diseases eventually, and unexpectedly, resulted in the discovery of a new class of subviral agents, the viroids. Their properties are consistent with those predicted for the first minimal replicons that populated the RNA world, bolstering the case for its existence.

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The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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